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Integrated RNA- and protein profiling of fermentation and respiration in diploid budding yeast provides insight into nutrient control of cell growth and development

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Short title: Integrated genomics analysis of yeast fermentation and respiration

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Abstract

Diploid budding yeast undergoes rapid mitosis when it ferments glucose, and in the presence of a non-fermentable carbon source and the absence of a nitrogen source it triggers sporulation. Rich medium with acetate is a commonly used pre-sporulation medium, but our understanding of the molecular events underlying the acetate-driven transition from mitosis to meiosis is still incomplete. We identified 263 proteins for which mRNA and protein synthesis are linked or uncoupled in fermenting and respiring cells. Using motif predictions, interaction data and RNA profiling we find among them 28 likely targets for Ume6, a subunit of the conserved Rpd3/Sin3 histone deacetylase- complex regulating genes involved in metabolism, stress response and meiosis. Finally, we identify 14 genes for which both RNA and proteins are detected exclusively in respiring cells but not in fermenting cells in our sample set, including *CSM4*, *SPR1*, *SPS4* and *RIM4*, which were thought to be meiosis-specific. Our work reveals intertwined transcriptional and post-transcriptional control mechanisms acting when a *MATa/α* strain responds to nutritional signals, and provides molecular clues how the carbon source primes yeast cells for entering meiosis.

Significance

Our integrated genomics study provides insight into the interplay between the transcriptome and the proteome in yeast cells undergoing vegetative growth in the presence of glucose (fermentation) or acetate (respiration). Furthermore, it reveals novel target genes involved in these processes for Ume6, the DNA binding subunit of the conserved histone deacetylase Rpd3 and the co-repressor Sin3. We have combined data from an RNA profiling experiment using tiling arrays that cover the entire yeast genome, and a large-scale protein detection analysis based on mass spectrometry in diploid *MATa/α* cells. This distinguishes our study from most others in the field - which investigate haploid yeast strains - because only diploid cells can undergo meiotic development in the simultaneous absence of a non-fermentable carbon source and nitrogen. Indeed, we report molecular clues how respiration of acetate might prime diploid cells for efficient spore formation, a phenomenon that is well known but poorly understood.

Introduction

Budding yeast is an important model organism for biochemical studies, genetic analyses and genomic profiling of cell growth and division, and cell differentiation [1-3]. In addition, it is useful for the field of toxicogenomics, which investigates the cellular response to environmental stressors and drugs; [4, 5], reviewed in [6].

S. cerevisiae was the first eukaryote for which the complete genome sequence was determined (see Saccharomyces Genome Database, SGD) [7, 8]. The protein-coding part of the yeast genome includes *verified genes*, which are conserved open reading frames (ORFs) typically associated with a biological process, *uncharacterized genes*, which play no known roles, and *dubious genes*, which are assumed not to encode functional proteins [8]. The yeast transcriptome has been studied in numerous conditions using microarrays and RNA-Sequencing (RNA-Seq) [9-12]. Furthermore, protein-profiling experiments detected nearly all theoretically predicted yeast proteins in pre-fractionated extracts from growing cells [13, 14]. Very recent feasibility studies reported vastly improved experimental protocols yielding quantitative information about protein concentrations in total cell extracts [15-17].

To produce the metabolite adenosine triphosphate (ATP), which is critical for storing chemical energy, yeast cells process fermentable and non-fermentable carbon sources by activating distinct metabolic pathways. Fermentation of sugars such as glucose and fructose is efficient and down-regulates enzymes required for metabolizing alternative carbon sources via a process called carbon catabolite repression; reviewed in [18]. Fermentation leads to the accumulation of glycerol and ethanol, which are further processed via oxidation. When yeast cells are cultured in rich medium in the presence of the two-carbon compound acetate, they use the tricarboxylic acid (TCA) cycle, and the glyoxylate cycle for energy production [19, 20]. Previous analyses of yeast cell growth under highly controlled conditions limited for carbon, nitrogen, phosphorus and sulphur revealed that both transcriptional control and post-translational regulation mechanisms play roles in the response to nutrient starvation [21-23]. Fermentation and respiration are not only relevant for understanding yeast growth and development but also for human diseases; for example, the so-called Warburg effect has been observed in tumour cells that excessively ferment glucose into lactate [24].

Ume6 is the DNA binding subunit of two conserved repressor complexes including either the histone deacetylase (HDAC) Rpd3 and the co-repressor Sin3 [25, 26], or the chromatin-remodeling factor Isw2 [27]. Ume6 was initially identified as a mitotic repressor for early

meiosis-specific genes, and later shown to orchestrate metabolic functions, stress response, and the onset of meiotic differentiation [28-30]. Among others, Ume6 directly represses *SIP4*, which encodes a C6 zinc cluster transcriptional activator involved in gluconeogenesis, during growth in the presence of glucose [30, 31]. Ume6 becomes unstable when vegetatively growing cells switch from metabolising glucose to processing acetate, and is temporarily degraded during meiosis by the anaphase promoting complex/cyclosome (APC/C), before it accumulates again at later stages of spore formation [32, 33].

In earlier work we have developed *Direct Iterative Protein Profiling* (DIPP) to comprehensively analyse the proteome of fermenting diploid yeast cells in a cost-effective manner by widely available standard mass spectrometry [34]. In the present study we report the changes in the proteome of diploid *MATa/α* yeast cells undergoing logarithmic growth in rich medium with fermentable glucose (YPD) or non-fermentable acetate (YPA) in the context of corresponding RNA-profiling data from wild-type cells and a *ume6* mutant strain. This approach enabled us to gain insight into molecular events that underlie the switch from fermentation to respiration, and that prime diploid *MATa/α* cells for the transition from mitotic growth to meiotic differentiation.

Materials and Methods

Culture conditions for protein profiling

Samples from YPD (yeast extract, peptone and dextrose) were cultured as published [34]. In addition, cells grown in YPD were transferred into 100 ml YPA (rich medium with acetate) pre-warmed to 30°C at a cell density of 2×10^6 cells/ml and cultured until they reached 3×10^7 cells/ml, before they were harvested in two 50 ml aliquots and washed with sterile water. The pellets were then snap-frozen in liquid nitrogen and stored at -80°C.

Construction of gene deletion strains

To reduce the chance of unlinked mutations affecting the growth experiments, both copies of target genes were deleted in homozygous diploid cells. Most deletions were made in the BY4743 background using the method as published [35]. Three deletions were made in the related strain JHY222 [36].

Plate growth assays

To monitor the growth phenotype of deletion strains we used two methods. Homozygous diploid strains were streaked on YD/G418 and grown at 30°C for three days. Single colonies from each strain were suspended in water and diluted to approximately 100 cells/ μ l. 4 μ l of cell suspension were spotted onto YPD and YPEG (ethanol, glycerol) plates and incubated at 30°C for two days (YPD) and three days (YPEG). Alternatively, strains were re-streaked for single colonies on YPD, YPA and YPEG plates, and incubated at 30°C for three days (YPD) or four days (YPA, YPEG).

Protein detection by immunofluorescence

Rim4 immunostaining was done as described [37]. Briefly, cells were fixed in 70% ethanol for one hour at room temperature, washed with PBS and incubated for 60 minutes at 37°C with a mouse anti-V5 monoclonal antibody (Invitrogen, USA) diluted 1:200 in 1x PBS 0.1% Tween 20. Cells were washed three times for 3 minutes each with 1x PBS 0.1% Tween 20 and incubated with a secondary Alexa Fluor 488 Goat Anti-Mouse antibody (Invitrogen)

diluted 1:10000 in 1x PBS. Cells were again washed and mounted in Antifade solution (Vector Laboratories) containing 4',6-diamidino-2-phenylindole (DAPI; 1 µg/ml) to stain DNA. Acquisition of images was done using a Zeiss AxioImager M1 microscope equipped with an AxioCam MRC5 camera controlled by AxioVision 4.7.1 software using standard settings (Zeiss, Le Pecq, France).

Chromatin immunoprecipitation (ChIP) assay

Ume6/DNA complexes formed *in vivo* were precipitated using a standard assay with SK1 cells cultured in rich medium (YPD). A 50 ml mid-log culture was cross-linked with 1% formaldehyde for 15 minutes at room temperature. Cross-linked Ume6/DNA complexes were quenched with 140 mM glycine (Sigma, USA) for five minutes, collected, washed and eluted before reversing crosslinks. Finally, DNA was precipitated, digested with proteinase K and amplified by Q-PCR as published. Relative ChIP signals were calculated using the formula $(2^{\Delta\text{-IP}} (\text{CT target} - \text{CT control}) / \text{input} (\text{CT target} - \text{CT control}))$. The *NUP85* coding region was used as an internal control [32, 38].

Protein extraction, sample pre-fractionation and peptide analysis by mass spectrometry

Duplicate samples from YPA were pre-fractionated, and the samples were analysed using DIPP as published [34]. Data were analysed using the Proteome Discoverer 1.2 software in conjunction with Mascot (Matrixscience) and SEQUEST database search engines to identify peptides and proteins. In the first step the MS/MS spectra were compared to annotation data for budding yeast proteins (Saccharomyces Genome Database release 06/01/2010) [8]. The mass tolerance for MS and MS/MS was set at 10 ppm and 0.5 Dalton. Trypsin selectivity was set to full, with one miss cleavage possible. The protein modifications were fixed carbamidomethylation of cysteines, variable oxidation of methionine, variable acetylation of lysine and variable phosphorylation of serine, threonine and tyrosine. Peptides identified were selected via Xcorr values and the Mascot score to achieve a false discovery rate of 1 % and a false positive rate of 5 %. We used Proteome Discoverer to assemble peptides detected in the first and second run, which are then masked in subsequent LC-MS/MS analyses. Before the third analysis step was executed, we combined peptide exclusion files from the first two runs. Peptides that were not masked were exported as a text file containing uncharged and accurate mass values (at five decimals) and a retention time window of approximately one minute. The

instrument was set to function with uncharged masses and to calculate the mass of a peptide based on its exact mass and charge state. A mass tolerance of ± 10 ppm was employed to filter out peptides already identified within the specified retention time window.

Minimum Information About a Proteomics Experiment (MIAPE) compliance

The mass spectra and search result data files are available for downloading at the Yeast Resource Center Public Data Repository (YRC PDR) via www.yeastrc.org/pdr/becke2014/ [39].

Culture conditions for RNA profiling

Triplicate samples of SK1 wild-type and *ume6* mutant strains were cultured in YPD, YPA and SPII using standard media and conditions [30, 40].

Probe synthesis, GeneChip hybridization and scan

RNA profiling with *Yeast 2.0* GeneChips was done as published [41].

*Differential expression analysis of wild-type versus *ume6* mutant strains*

To identify up-regulated genes in *ume6* mutant we first performed a quantile normalization and then selected genes for which the expression level was above the 25th percentile (9.006) in at least one condition, and that showed a fold-change > 2 between mutant and wild-type. A limma test with a False Discovery Rate (FDR) 5% was done to identify genes differentially expressed and up-regulated in the *ume6* mutant. The statistical filtration was done using R [42, 43].

Tiling array expression data integration

DNA-strand specific whole-genome expression data obtained with tiling arrays and duplicate samples from diploid SK1 cells cultured in rich medium (YPD) and rich medium with acetate (YPA) were integrated and compared to the mass spectrometry measurements; data processing methods and expression threshold level parameters were as published [36]. For each gene annotated in the reference genome, we selected the segments derived from Sc_tiling experiments, which overlapped its coordinates, and computed an average value for

the gene. Therefore, each segment's contribution to the overall signal is proportional to its overlap with the corresponding gene. Equivalent values were computed for overlapping antisense transcripts for each gene. Genes were organized into 11 clusters, and for each gene (as represented by segments) we extracted the list of the clusters it was associated with.

Microarray expression data

Expression signals were obtained with Affymetrix DNA-strand specific tiling arrays (Sc_tlg) from reference [36].

Regulatory motif prediction

The URS1 motif was predicted using the published Promoter Analysis Protocol (PAP) [44].

Global genomics data integration

Information about protein and genes and the Gene Ontology annotation data were downloaded from SGD [8]. The relative abundance data of proteins expressed in log-phase growth were extracted from the quantitative Western blot analysis of TAP-tagged strains available via SGD (<http://yeastgfp.yeastgenome.org/>) [45]. Phosphorylated sites predictions were extracted from [46]. Genome annotation data for haploid S288c and SK1 strains were used as published [34].

Identifying target genes showing a similar pattern as the reference genes

We filtered genes similar to *KGD1*, *MDH1*, *MDH2*, *SDH1*, *FUM1*, *IDH1*, *ACO1*, *CIT1*, *MLS1*, *ICL1* whereby (i) the target genes were expressed above the background (BG)=5.99 in YPD and YPA, (ii) the fold change between expression in YPD and YPA was >2.0, (iii) the log-expression in YPA was >12, and (iv) we observed ≥ 3 -fold more bands to contain the protein in YPA than YPD.

Gene Ontology enrichment analysis

We performed the GO enrichment analysis with the GOToolBox [47], using an hypergeometric law and a Benjamini and Hochberg correction for multiple testing. Biological Process GO annotations with corrected p -values <0.01 were selected.

Identification of proteins detected specifically in YPA

We selected proteins for which peptides were found only in YPA but not YPD classified the genes, which encode them into loci, which are not significantly differentially expressed in YPD and YPA, which are differentially expressed in YPD and YPA, with a fold-change >1.5 as assessed by a linear models for microarray data (LIMMA) statistical test with FDR=5%, and genes, which are expressed $<BG=5.99$ in YPD and $>BG=5.99$ in YPA, with a fold-change >1.5 as assessed by a limma statistical test with FDR=5% [42, 43].

Results

Experimental design

To determine the proteome in the presence of glucose or acetate we combined previously published DIPP data on rapid growth in rich medium with glucose (YPD) [34], with a new dataset from cells cultured in a medium where glucose was replaced by acetate (YPA). We included published RNA profiling data from YPD and YPA samples that were analyzed with tiling arrays (Sc_tlg) [36]. We previously reported that *SIP4*, *CAT8* and *ADR1* - which encode transcription factors involved in gene expression during the diauxic shift - are strongly repressed in a *ume6* mutant (GeneChip expression data are shown in www.germonline.org; [48]) [49]. Since a large-scale *in vivo* protein-DNA interaction study showed that Ume6 binds to their promoters (ChIP-Chip *in vivo* binding data are shown in www.germonline.org) [31], we investigated the role of the Rpd3/Sin3/Ume6 regulatory complex in respiring diploid cells. To this end, we used expression data from *UME6* wild-type cells and a *ume6* mutant strain (Lardenois, Walther, Becker et al., in preparation; the complete dataset will be published elsewhere). The transcriptome and proteome data were then integrated with functional genomics data [4, 8, 50]. To allow for direct comparison with previous studies, we have employed standard flask culture conditions for asynchronous cell division in rich media with

glucose or acetate – the latter is used as a non-fermentable carbon source in pre-sporulation growth medium [40, 51, 52] – and cell differentiation in sporulation medium.

The proteome of diploid budding yeast during fermentation and respiration

Like in earlier work on the proteome of cells cultured in YPD [34], we found that protein size and migration speed in the gel - that is, protein presence in gel slices 1 (top) to 30 (bottom) - are correlated similarly across YPA replicates (Figure 1A). We also observed that the number of bands a protein was present in was highly reproducible in both media (correlation coefficient r^2 0.898 in YPD and 0.877 in YPA; Figure 1B).

By combining data obtained with cells cultured in rich media with glucose or acetate we detected 5513 out of 6713 predicted proteins in at least one of the four samples (82%, taking verified, uncharacterized and dubious genes into account). More specifically, we found 4517 of 4877 proteins encoded by verified genes (93%; Table 1, Figure 1C). Among 4952 proteins detected in YPD, 3520 were found in both samples, while 883 and 549 were detected only in YPD1 or YPD2, respectively. Likewise, among 5020 protein scored as present in YPA, 3733 were reproducibly detected, while 655 and 632 were present only in replicates 1 or 2 (Figure 1D). As expected, the vast majority of verified ORFs encode proteins, which are expressed highly enough to be detected in almost all samples, while the output among uncharacterized genes is split into two major groups that are either reproducibly detected, or that are undetectable in the experimental conditions used (Table 2, Figure 1E).

In the case of dubious ORFs we detect few proteins, which is in keeping with the notion that many of these loci are annotation artefacts. However, we note that 20 proteins encoded by dubious ORFs are detected in all four YPD/YPA samples (Figure 1E, Supplemental file S1, Class 1 detection pattern, filter for “dubious”). Among them, Yjr146w was detected in an earlier protein profiling study [45] and two other proteins may be involved in cell aging (Yim2 [53]) or drug-resistance (Imd1 [54]). For eight dubious ORFs we also detect corresponding mRNAs: *YMR173W-A* almost entirely overlaps *DDR48*; weak expression of mRNA was observed for *YBL071C*, *YCR013C*, *YHR056W-A*, *YLR339C*, *YMR075C-A* and *YPR197C* and *YMR304C-A* appears to be co-expressed with the partially overlapping *SCW10* locus. In the cases of *YAL016C-B*, *YBR191W-A*, *YKL165C-A*, *YLR163W-A*, *YMR052C-A*, *YNL319W* and *YPR136C* no expression signals were observed with tiling arrays; these cases

are either false positive peptide scores or the transcript levels are below the threshold level of detection of tiling arrays.

Among approximately 1000 proteins known to localise to mitochondria we detect the majority (718) in all samples; notably, 57 proteins are scored as present only in cells grown in YPA (including 31 that are detected in both YPA replicates). To the contrary, only 9 proteins were found exclusively in cells cultured in YPD (Figure 1F).

We conclude that DIPP detects the vast majority of known yeast proteins and that growth in acetate increases the protein content of mitochondria in accordance with their metabolic role in respiration [55-57]. Finally, the group of dubious ORFs may represent an opportunity for the discovery of genes involved in vegetative growth.

Comparing expression levels and protein detection patterns in diploid *MATa/α* cells

We subsequently asked if DIPP's capacity to detect a protein was related to the overall expression level of the corresponding gene, and found that the median intensities of log-transformed microarray expression signals increase with the number of samples in which the proteins are detected by DIPP in cells cultured in YPD or YPA (Figure 2A). Gene expression values are also positively correlated with the number of bands a protein is detected in, and the average number of peptides identified (Figure 2B, C). To further correlate gene expression and protein function, we compared protein profiles in YPD and YPA media with mRNA concentrations during fermentation (YPD), respiration (YPA) and sporulation (SPII hourly time points from 1 to 12) using our published tiling array data from SK1 *MATa/α* wild-type cells [36]. Coherently, we found that proteins encoded by genes showing peak expression in cells undergoing mitotic divisions tend to be reproducibly detected in one or both growth media (Figure 2D, patterns 1-4). On the contrary, proteins encoded by genes for which mRNA synthesis is induced during early, middle and late stages of meiosis and gametogenesis, are not - or at least not reproducibly - detected in mitotic cells (Figure 2D, patterns 5-10). It is unclear why in the case of very late sporulation genes the DIPP pattern is slightly shifted toward protein detection again, perhaps it reflects the fact that certain proteins play roles during mitotic growth and spore formation (Figure 2D, pattern 11).

Transcriptional regulation versus post-transcriptional control during fermentation and respiration

We first asked if proteins important for metabolizing acetate showed correlated profiles of mRNA induction and protein accumulation. To address this question, we determined the mRNA/protein patterns underlying the switch from processing glucose to metabolizing acetate in the cases of genes essential for the process. We selected 10 reference proteins required for respiration, and found them both in fermenting and respiring cells (Figure S1A) [8]. Subsequently, we selected 60 genes, which show RNA/protein patterns similar to the reference genes (Figure S1B). Gene Ontology terms enriched in this group point to metabolic processes (*cellular respiration*, p -value 1.45×10^{-7} ; *carboxylic acid metabolic process*, 1.24×10^{-24}), and a response to nutritional cues (*nitrogen compound biosynthetic process*, 3.52×10^{-7} ; Figure S1C). We next assayed genes involved in mitochondrial inheritance and function (*AIM41*, *YCP4*), the TCA cycle (*LSC2*), ethanol metabolism (*ADH2*), iron transport (*FTH1*), cell structure (*CWPI*), stress response (*CMK2*, *LSB3*, *YJR085C*), and possibly respiration (*Ypr010c-a* via interaction with Fcj1[58]) for growth on a non-fermentable carbon source. However, none of them failed to grow on YPA and YEPG media after incubation at 30°C; *FUM1*, *ACO1*, *OMI4* and *PTH1* were used as positive controls (Figure S1D). We conclude that proteins important for growth on acetate are not necessarily present only in respiring cells but already accumulate during fermentation.

Ume6 coordinates the transition between cell growth and development, and both its concentration and DNA binding activity decrease when cells are cultured in acetate [30, 32, 59, 60]. To learn more about the transcriptional control mechanisms that contribute to the mitotic regulation of genes differentially expressed during fermentation and respiration we combined URS1 motif predictions with genome-wide expression data from wild-type cells and *ume6* mutant cells. We find that likely direct Ume6 targets are induced in YPA. This comprises six genes for which promoters contain known URS1 motifs (*ACH1*, *ACS1*, *CAT2*, *GDH2*, *PRX1*, *UBI4*), and three genes that have promoters containing predicted URS1 elements (*ADY2*, *PRB1*, *OMI4*; Figure S1B and Supplemental file S1). *ADY2* encodes a membrane bound acetate permease essential for the packaging of meiotic nuclei into four spores [61, 62]. The latter function is apparently specific for Ady2 since no meiotic phenotype was reported for its paralog Ato2 [63]. To validate our motif predictions and the RNA profiling data we confirmed that Ume6 binds to the promoters of *ACH1* and *ADY2* *in vivo* using chromatin immunoprecipitation assays. The binding signal intensities were found to be

in the same range as the Ume6 targets *CAR1* and *HOP1* ([28, 64, 65]; Figure S1E). Furthermore, *ume6* mutants in three different genetic backgrounds fail to grow normally on YPA and - in the case of W303 – display increased cell size (Figure S1F, G) [29]. These findings extend the metabolic role of the Rpd3/Sin3/Ume6 regulator as diploid cells switch from fermentation to respiration.

Protein profiling reveals that carbon-source specificity is biased toward acetate

We next sought to distinguish genes that are transcriptionally controlled by Rpd3/Ume6, from those that are regulated at the post-transcriptional level when diploid cells switch from fermentation to respiration. We therefore assembled a list of 94 proteins detected only in YPD and 169 proteins found only in YPA and then used tiling array data to filter three groups of YPD/YPA mRNA patterns in each medium.

The first YPD-specific group includes 56 genes for which we detect a gradient of similar concentrations (that is, the differences are not statistically significant) in both YPD and YPA (Figure 3A). This group contains Spo23 (a Spo1 interactor [66]) and Mum3 (spore wall formation [67]), for which weak mRNA signals are detected in YPD [36], and five genes up-regulated in the *ume6* mutant (*BSC2*, *MCH1*, *SDT1*, *YNL155W*, *ZPS1*).

The second YPD-specific group comprises 17 genes for which mRNAs coherently peak in rich medium (Figure 3B). We note the presence of supposedly sporulation-specific Smk1 (MAP kinase required for spore wall assembly [68]) in this group. We also find another gene showing elevated levels during growth in the absence of Ume6 (*DIF1*).

The third YPD-specific group covers 21 genes for which mRNAs peak in YPA (Figure 3C). This pattern clearly indicates that transcription and translation and protein stability are uncoupled for these loci. Again, we detect a protein considered to be sporulation-specific (Sps100, involved in spore wall formation [69]), for which tiling array data show clear expression in dividing cells [36].

The first YPA-specific group includes 58 genes for which we observe similar mRNA levels across media (Figure 4A). Among the proteins that accumulate in YPA we found Spo21, which is mainly involved in meiosis specific modification of the spindle pole body, but for which a function in nutrient utilization was also reported [70, 71], and Sae2, a protein involved in DNA-damage response and meiotic double strand break repair [72, 73]. This

group also contains *MET30*, *STS1* and *YLR194C*, which harbour predicted URS1 motifs in their promoters and are partially de-repressed in acetate in the absence of Ume6 (Figure 4A).

The second YPA-specific group comprises 97 loci for which mRNA is detected at various levels in YPD but its concentration increases in YPA (Figure 4B). URS1 motif predictions and RNA profiling data indicate that these genes include eleven new potential Ume6 targets involved in acetate transport and spore packaging (*ADY2*), respiration (*RSF1*), energy metabolism (*BNA2*), cell wall integrity (*PUN1*), protein transport (*VPS73*), protein turnover (*DAS1*), protein modification (*PIG1*), thermotolerance (*NTH2*), DNA repair (*PDC1*), multi-drug transport (*PDR10*), and stress response (*YLR446W*). Interestingly, we also find *RIM8* and *RIM13*, which control *RIM1*, a positive regulator of sporulation and invasive growth [74, 75], and *IMP2*, a gene required for respiration and sporulation [76].

The third YPA-specific group consists of 14 genes for which mRNAs and proteins are detected only in YPA (Figure 5A). Some gene functions are coherent with growth in YPA, such as energy metabolism controlled by glucose and ethanol (*ICL2*), stress-induced protein transport (*YPT53*), stress response (*SPL2*), carbohydrate transport (*MPH3*), glycerol transport (*STL1*), sulphur catabolism important for efficient anaerobic growth (*JLP1*), amino acid biosynthesis (*STR3*), and fatty-acid oxidation (*POT1*). Furthermore, we identified a *dubious* gene (*YDR510C-A*), a gene involved in cell fusion during mating (*FUS1*), and another four genes previously considered to be meiosis-specific (*CSM4*, *RIM4*, *SPR1* and *SPS4* [77-82]). To find out if genes showing such patterns played new roles during respiration, we used a plate growth assay to analyse diploid homozygous *csm4*, *rim4*, *sps4*, *stl1* and *str3* mutants in the presence of fermentable- or non-fermentable carbon sources. None of the mutants appeared to be respiration deficient. *HO* and *FAR3* were used as negative controls, and *ACO1* and *MRM1* were positive controls (Figure 5B). The third group includes three genes with known URS1 motifs (*CSM4*, *SPI2*, *STR3*) and two new targets that contain predicted Ume6 binding sites in their upstream regions (*POT1*, *RIM4*). Rim4 is an RNA-binding protein important for mRNA translation during meiosis and spore formation [78, 79]. Since the Ume6 level decreases and its ability to bind DNA diminishes when cells enter respiration, it is possible that *RIM4* is partially de-repressed in YPA [32, 59, 60]. To rule out that Rim4 accumulates in YPA because the protein is strongly induced in a small sub-population of cells undergoing premature sporulation [77, 78], we analysed a strain expressing C-terminally tagged Rim4 (which showed normal growth and sporulation properties), and found that all cells cultured in YPA expressed the protein (Figure 5C).

Discussion

We integrated transcriptome and proteome data from growing diploid *MATa/α* yeast cells cultured in rich medium, and pre-sporulation medium where glucose was replaced by the non-fermentable carbon source acetate. We interpreted the output in the context of functional information. Our results contribute to yeast genome annotation, and they help gain insight into the molecular processes, which coordinate energy metabolism with stress response during cell growth and differentiation.

Why are proteins absent although the cells contain their cognate mRNAs?

For 1630 ORFs we find evidence for mRNAs but not for proteins in at least one medium (some proteins in this category also fall into the class of 5513 proteins detected in at least one medium, which is why their sum is greater than the total number of 6713 predicted proteins). The proteins in this group tend to be somewhat smaller than the global average (median of 231 amino acids versus 358 for all proteins; *p*-value of Wilcoxon rank sum test with continuity correction $< 2.2 \times 10^{-16}$; Figure S2A). 598 proteins are less than 150 amino acids long, and may therefore have been lost during the pre-fractionation procedure, but for 1032 cases other explanations such as annotation artefacts, special biological roles or inherent instability during growth seem more likely. Indeed, among 1032 ORFs we find 47 *dubious* genes and 200 *uncharacterized* genes. Furthermore, significantly enriched terms from the *Biological Process* Gene Ontology reveal functions likely not important during fast mitotic growth in rich media: this includes processes related to retroviruses and gametogenesis such as *transposition*, *RNA-mediated* and *transposition* (*p*-value: 6.28×10^{-32}), *reproduction* (7.38×10^{-5}), *reproductive process* (3.85×10^{-5}), and *DNA recombination* (2.08×10^{-8}). Other pathways are *DNA metabolic process* (0.00141), *protein catabolic process* (0.00264), *proteolysis* (0.00400), *regulation of biological quality* (0.00868), and notably, *response to chemical stimulus* (0.00343), reflecting in part the activity of stress-response genes likely repressed during growth under perfect conditions (Figure S2B).

Gene expression and protein function

We identified two genes as potentially important for fermentation (*YJR085C*, *YPR010C-A*), which were not among the loci studied by systematic gene deletion experiments and chemoprofiling [50, 83]. Strain lacking these genes did, however, not display any discernible

growth phenotype in plate assays using ethanol/glycerol or acetate as non-fermentable carbon sources (Figure S1C). Since the genes have no obvious paralog, genetic redundancy cannot explain this result. Given that Yjr085c is a mitochondrial protein induced by chemically induced DNA damage, it is possible that its role is limited to a stress response other than processing a non-fermentable carbon source [55, 57, 58, 84]. Likewise, Ypr010c-a interacts with, among others, Ubi4 (protein degradation) and Nab2 (mRNA export), which are involved in DNA replication stress [58, 85, 86]. Yjr085c and Ypr010c-a may therefore exert their activities only under experimental conditions unrelated to carbon metabolism.

Nutritional control of cell fate involves the HDAC Rpd3/Sin3/Ume6 complex

We identified 14 loci for which we detected both mRNA and proteins exclusively in YPA. This group includes four cases - *CSM4*, *SPR1*, *SPS4*, and *RIM4* - thought to be active only in sporulating cells. It is unclear why previous studies did not observe these proteins in dividing cells as well, but it might be due to cell culture protocols and strain backgrounds. Csm4 localizes to the nuclear envelope and is required for the mobility of telomeres during meiosis, and Sps4 was initially reported to be a meiosis-specific gene induced during middle sporulation [80, 82]. In SK1, we find that the *CSM4* and *SPS4* are weakly transcribed in YPA and strongly induced when cells have initiated meiosis (SGV; www.germonline.org). This low level of expression might be enough for the proteins to accumulate. The detection of Spr1 in YPA is unexpected because the protein acts during late sporulation where it renders spores thermoresistant [87]. Moreover, tiling array data show that *SPR1* is only partially expressed in YPA from what appears to be an internal transcription start site (SGV via www.germonline.org), and it is possible that the overlapping antisense lncRNA SUT785 contributes to *SPR1*'s mitotic repression. Perhaps the fortuitous expression of a shorter mRNA or mRNA levels below our threshold level of detection are sufficient to allow for the accumulation of a truncated or a full-length version of Spr1 in YPA. *RIM4* is induced in YPA as opposed to an earlier report where neither mRNA nor protein were detected in this medium [77]. Both studies used the SK1 strain and YPA pre-sporulation medium but work by Soushko and Mitchell was done in an SK1-derived background lacking the *GAL80* regulator, which somehow may influence *RIM4* expression. The upstream region of *RIM4* contains URS1 motifs and was found to interact with Ume6 *in vivo* [31, 77]. Moreover, we observe that the *RIM4* mRNA is de-repressed in *ume6* mutant cells cultured in YPD and YPA. While the data imply that *RIM4* is transcriptionally controlled like a typical early meiotic gene [88],

our results beg the question why *RIM4* is induced in YPA when the Rpd3/Sin3/Ume6 repressor complex is still active. It is known that acetate triggers Ume6's degradation and negatively influences its ability to bind DNA, which perhaps enables Rim4 to accumulate during vegetative growth in respiring cells (at least in a genetic background that sporulates efficiently) [32, 59, 60]. It is not clear why Rim4 accumulates in YPA, because it does not seem to be involved in producing energy with a non-fermentable carbon source (see Figure 4B). However, the protein is required for the complex and highly stage-specific translational control of many proteins during meiosis via binding to 5'-UTRs, including some that are important for M-phase [79]. We therefore speculate that Rim4's early accumulation in respiring cells during late-log phase enables the SK1 background to rapidly progress through the meiotic differentiation pathway once it is triggered by the onset of Ime1 expression [2].

We have recently reported that the Rpd3/Sin3/Ume6 complex represses early meiotic transcript isoforms with extended 5'-UTRs during vegetative growth [89]. This, together with the observations reported here, raises the interesting possibility that the progressive degradation of Ume6 during the switch from fermentation to respiration and sporulation, simultaneously de-represses the 5'-UTR interacting protein Rim4 and long transcript isoforms that are bound by the protein [79].

Conclusion

The present study reveals complex regulatory processes affecting mRNA and protein stability that underlie the transition from fermentation to respiration in sporulation-competent diploid yeast cells. Moreover, it provides further insight into the role of the conserved HDAC complex Ume6/Rpd3/Sin3 in coordinating metabolic functions with stress response and cellular differentiation in a simple eukaryote. Given the recent major advances in proteomics [17] the next goal will be to determine the comprehensive proteome of the entire diploid yeast life cycle, and to integrate this information with the transcriptome, functional annotation data and protein networks.

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Figure legends

Figure 1. The proteome of fermentation and respiration. (A) Protein length in amino acids (y-axis) is plotted against the gel slice number (x-axis) for the color codes samples as shown (B) The number of proteins reproducibly found in gel slices 1-30 are plotted. (C) Pie charts show the percentages of proteins encoded by all open reading frames (ORFs) or verified ORFs for riche medium with glucose (YPD) in grey, for rich medium with acetate (YPA) in red, and for the total number of proteins in violet. (D) Venn diagrams show the numbers of proteins detected in sample replicates. (E) Color coded bubble charts show the patterns of detection (0 means not detected, 1 signifies that the protein was detected in one replicate, 2 means that it was detected in both replicates) in YPD (y-axis) versus YPA (x-axis). Data are given in grey for all ORFs, in green for verified ORFs, in blue for uncharacterized ORFs, and in red for dubious ORFs. (F) A bubble chart shows the detection pattern in YPD replicates (x-axis) versus YPA samples (y-axis) for proteins known to localize to mitochondria like in panel E.

Figure 2. The correlation between gene expression and protein detection. (A) A color-coded bar diagram shows median log microarray signal intensities (y-axis) for proteins that were not detected (0), detected in one replicate (1), or detected in both replicates (2) in YPD and YPA (x-axis) in grey and red, respectively. An orange line shows the background (BG) threshold signal. (B) log₂-transformed intensity values (y-axis) are plotted against the number of gel slices (x-axis) proteins were detected in for YPD (red) and YPA (blue). (C) A bar diagram shows the average log intensity (y-axis) versus the average number of peptides identified (x-axis) in YPD (grey) and YPA (red). The standard deviation is indicated. (D) A heatmap shows the microarray gene expression profiles (1-11) ordered over peak signals.

Log-transformed values are color-coded (blue indicates low signals and red indicated high signals). Rich media (YPD, YPA) and hourly time points in sporulation medium (SP11) are given at the top. The strain is indicated to the right. Color-coded bubble charts summarizing protein detection patterns are shown like in Figure 1E for 11 expression patterns as indicated.

Figure 3. Ume6-target gene profiling and YPD protein/mRNA detection patterns. (A) A color-coded heatmap indicates predicted URS1 motifs (grey squares), and de-repression in a vegetatively growing *ume6* mutant (up in *ume6Δ*). A green to pink color scale indicates gene expression measured by Yeast 2.0 in rich media (YPD, YPA), and three time points in sporulation medium (SP11 4 hours, 8 hours and 10 hours) in wild-type (SK1 *MATa/α*) versus *ume6* mutant strains (SK1 *MATa/α ume6*). The color change was set to be consistent with the tiling array expression background. A blue to red color scale indicates the expression level determined with tiling arrays in YPD and YPA. For proteomic data, the number of bands (from 0 to 30) within which a given protein was detected is summarized in bar diagrams given in light grey. GeneChip data (Yeast Genome 2.0), tiling array data (Sc_tlg), and mass spectrometry data (DIPP) are indicated at the bottom. Scales for Yeast Genome 2.0 (left) and Sc_tlg data (right) are given at the bottom. (B) Data are shown for selected genes that show similar patterns as the reference genes. (C)

Figure 4. Gene expression and Ume6-dependency for proteins detected only in YPA. (A) A color-coded heatmap is given like in Figure 3 showing proteins detected only in YPA, for which we observe a gradient of similar tiling array signals in YPD and YPA. (B) Examples of YPA-specific proteins encoded by genes for which we observe significantly different expression levels are shown. Scales are given at the bottom.

Figure 5. Correlating gene expression, protein detection and protein localization patterns. (A) A color-coded heatmap like in Figure 3 is shown for 14 target genes with an expression and a proteomic pattern specific for YPA. (B) The outcome of a plate patch assay on YPD (fermentation) versus YPEG (respiration) is shown for positive and negative controls in red and green, respectively and new targets given in black. (C) Immunofluorescence data are shown for tagged Rim4 (Rim4_{3V5}) in cells cultured in YPD and YPA. Cells were visualized using differential interference contrast microscopy (DIC). DNA and proteins were visualized using a fluorophor (DAPI) and an antibody against the tag (Rim4_{3V5}), respectively, and both are displayed together to detect nuclear signals and cytoplasmic signals (Merge).

Figure S1. Determining the growth properties of mutant strains. (A) A heatmap for ten manually selected reference genes indicates URS1 motifs (grey), and de-repression in a *ume6* mutant (up in *ume6Δ*). Samples were cultured in YPD, YPA and three time points in SPII (4h, 8h and 10h) in SK1 *MATa/α* versus *ume6* mutant strains. Scales for Yeast Genome 2.0 data (left) and *Sc_tlg* data (right) are given. (B) A heatmap is shown for genes similar to the reference genes like in panel A. (C) A bar diagram shows the annotation frequency for 13 Gene Ontology terms. The *p*-values are indicated. Light grey bars are for selected genes and dark grey bars are for reference genes. (D) The outcome of growth assays on solid media (YPD, YPA) is shown for selected target genes (*ADH2/YMR303C*, *AIM41/YOR215C*, *CMK2/YOL016C*, *CWP1/YKL096W*, *LSB3/YFR024C-A*, *LSC2/YGR244C*, *OM14/YBR2310C*, *PTH1/YHR189W*, *YCP4/YCR004C*, *YJR085C*, *YPR010C-A*), positive controls (*ACO1/YLR304C*, *FUM1/YPL262W*), and a negative control (*HO/YDL227C*). The duration of the growth assay is given in days below each photograph. Schematics indicate the position of each strain on the plate. (E) A bar diagram shows the fold-enrichment (y-axis) detected by chromatin immunoprecipitation for two target genes (*ACH1*, *ADY2*) versus three controls (*CAR1*, *HOP1*, *SPO13*; x-axis). (F) Differential interference contrast images are shown for wild-type and mutant strains as indicated at the top. The medium and culture conditions are given to the left. The strains are shown at the top. A white bar corresponds to 10 μm. (G) Plate assays for *ume6* mutants in three strain backgrounds indicated at the top a shown. The medium and culture conditions are shown to the right. A schematic indicated the positions of re-streaked wild-type colonies versus three independent mutant colonies.

Figure S2. (A) A color-coded bar diagram shows the number of amino acids in the primary sequence of proteins (y-axis) encoded by all ORFs (violet) or by ORFs not identified by proteomics for which we observe expression values greater than the background (BG=5.99, x-axis). The inter-quartile range (IQR, 25th and 75th percentiles) is indicated in the graphs in all panels. Wilcoxon rank sum test with continuity correction was used to assess median differences. (B) A bar diagram shows the percentage of genes associated with annotation terms for ORFs above expression background not identified by proteomics (dark grey) and all ORFs (light grey). *p*-values computed with hypergeometric law and corrected for multiple testing with B&H are indicated above the bars. Only Biological Process annotations with a *p*-value < 1% were selected.

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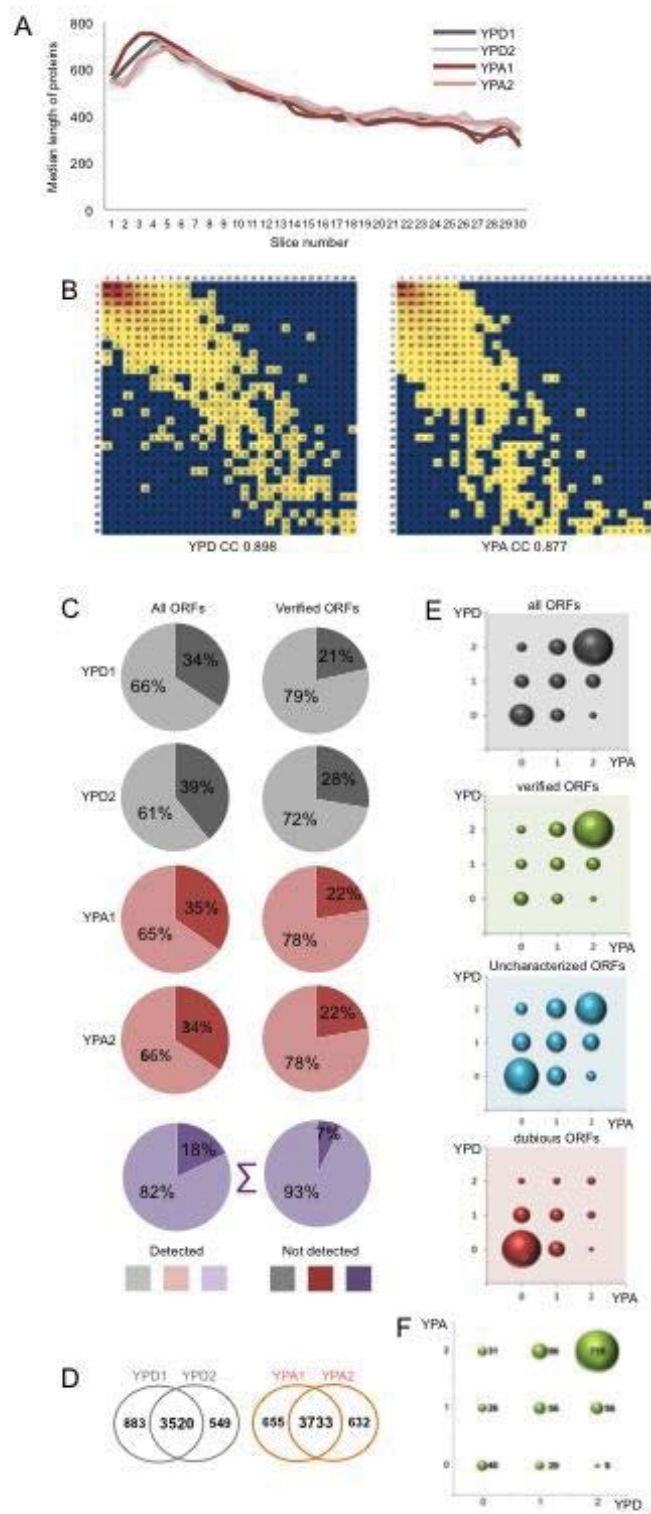


Figure 1

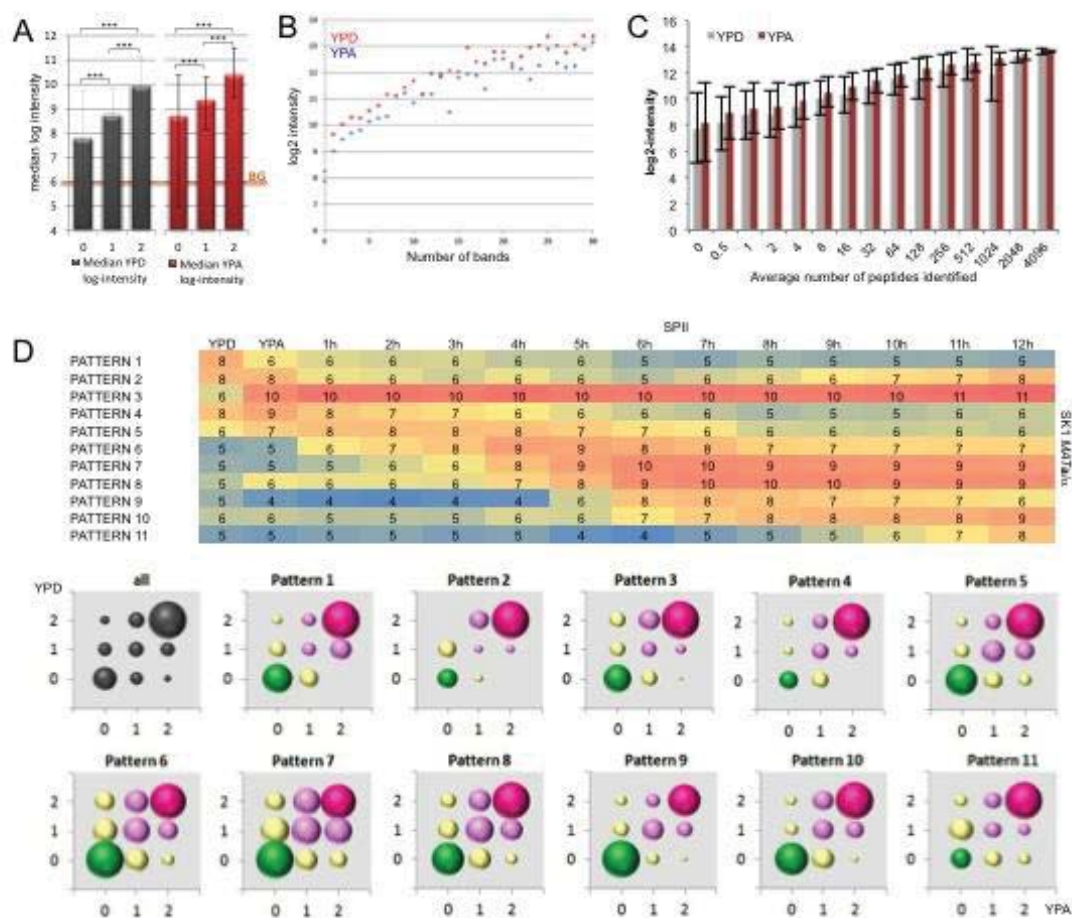


Figure 2

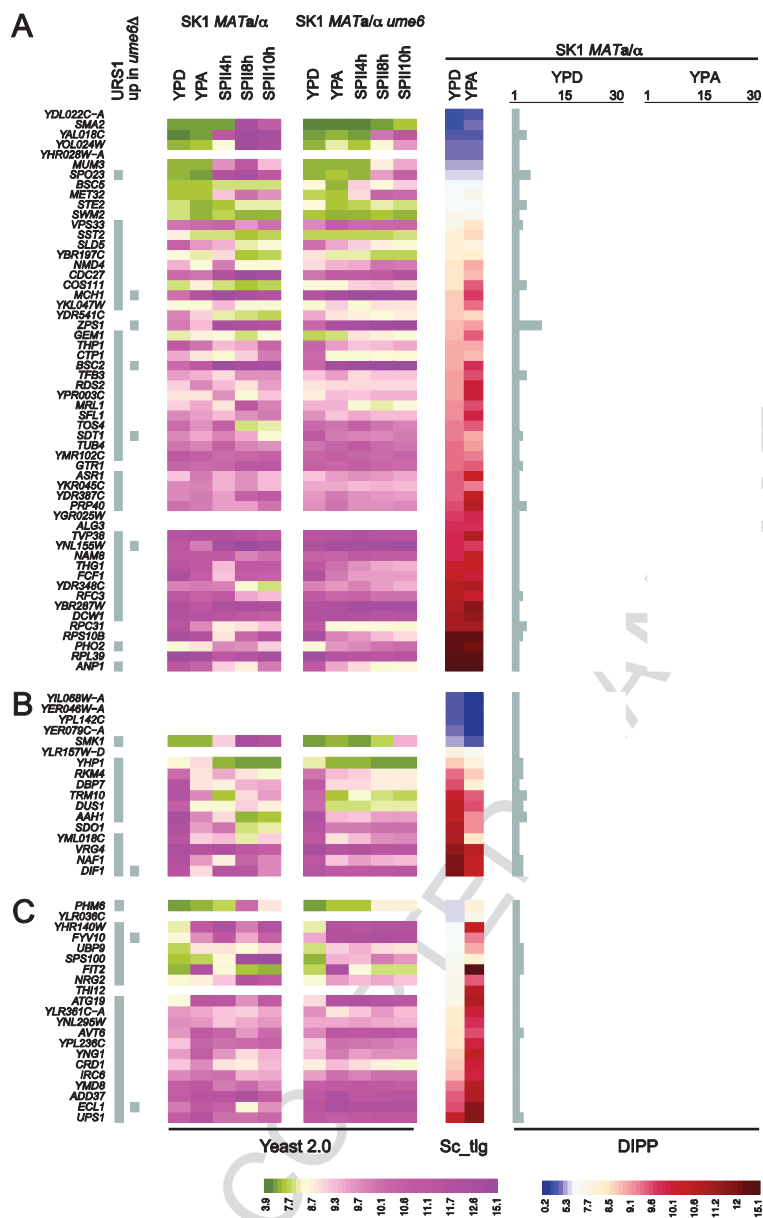


Figure 3

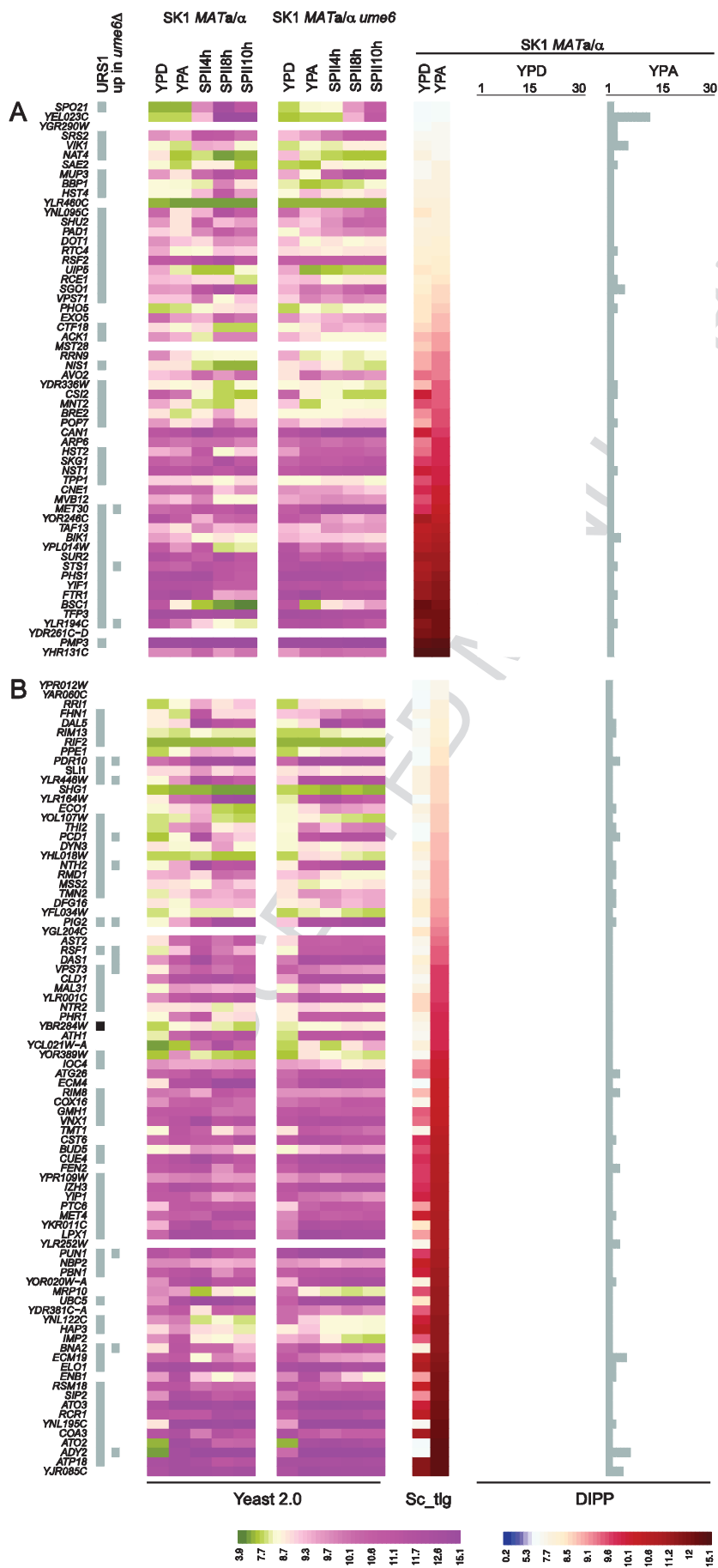


Figure 4

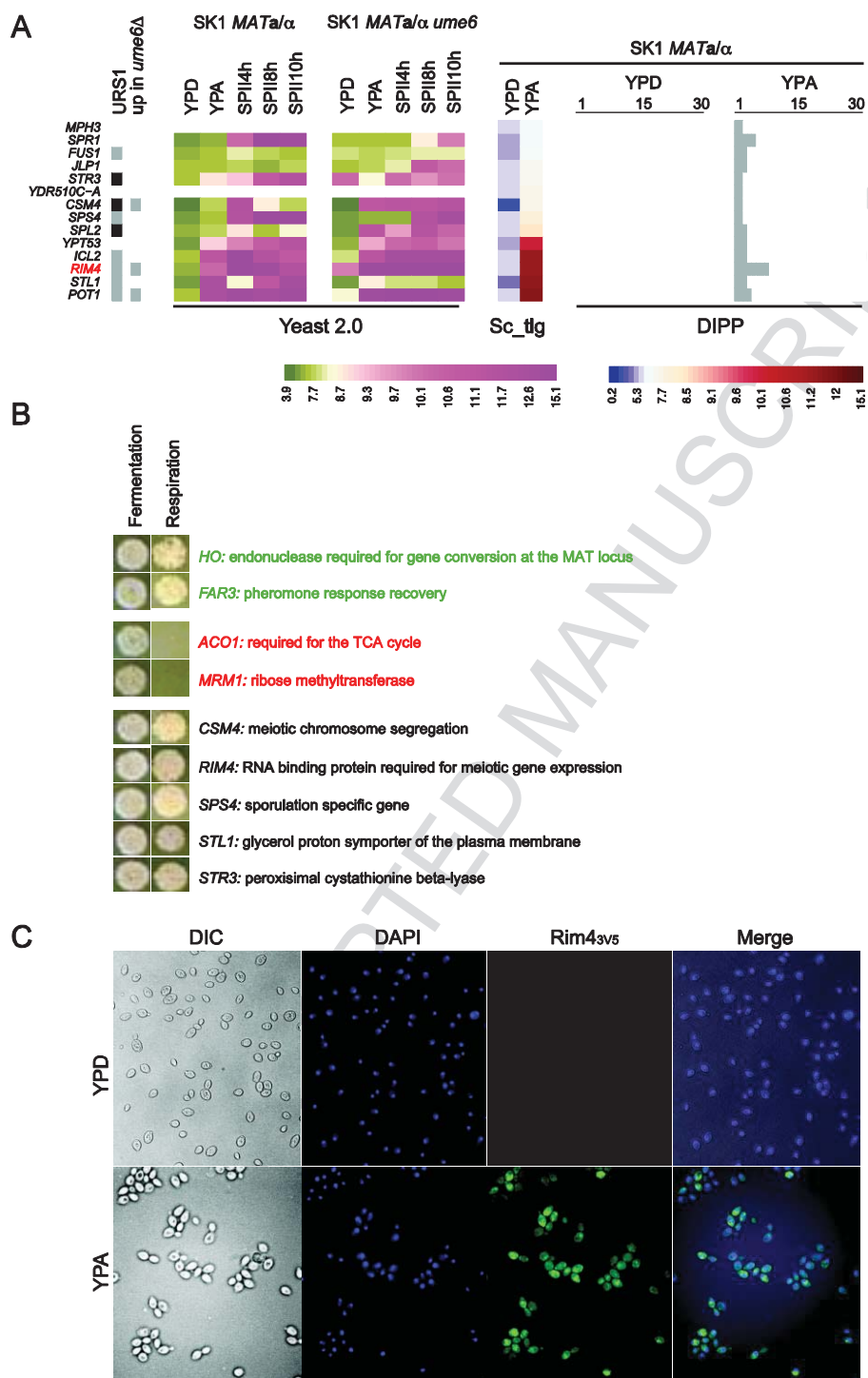


Figure 5

Table 1.

Annotation	YPD1	YPD2	YPA1	YPA2
All yeast ORFs	4416	4079	4379	4400
Verified ORFs	3833	3525	3793	3804
Uncharacterized ORFs	444	396	441	437
Dubious ORFs	117	139	126	142

Table 2

YPD	YPA	all ORFs	verified	uncharacterized	dubious
0	0	1090	356	266	491
0	1	368	198	74	90
0	2	175	131	30	13
1	0	381	207	77	85
1	1	504	335	85	59
1	2	547	446	82	16
2	0	94	67	20	7
2	1	415	331	61	20
2	2	3011	2769	215	20

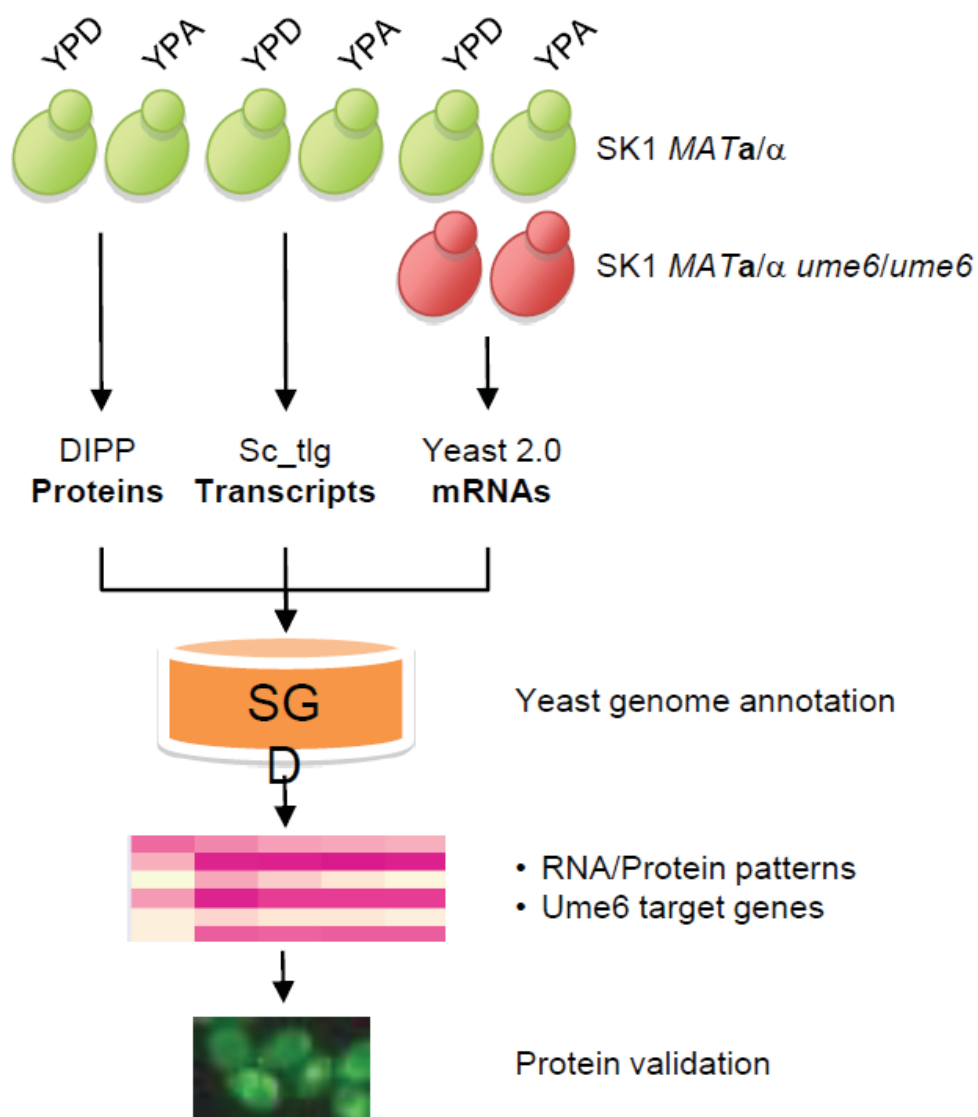
Table 3

YPD	YPA	average length (all ORFs)	average length (verified ORFs)	median length (all ORFs)	median length (verified ORFs)
0	0	234	309	126	247
0	1	308	376	212	335
0	2	416	443	370	412
1	0	327	393	229	346
1	1	388	434	349	417
1	2	482	502	416	447
2	0	361	389	338	353
2	1	471	496	422	451
2	2	576	581	468	476

Table 4

ORF	length (aa)	YPD (log2)	YPA (log2)	$2^{ YPA-YPD }$	N° slices in YPA	N° slices in YPA
<i>MDH2</i>	377	8.14	13.37	37.5	1.5	29
<i>ICL1</i>	557	8.19	13.81	49.2	17	29
<i>MLS1</i>	554	6.63	13.46	113.8	12	29.5
<i>SDH1</i>	640	10.07	12.83	6.8	17.5	26
<i>FUM1</i>	488	11.19	13.07	3.7	18	26
<i>ACO1</i>	778	12.01	13.17	2.2	27.5	30
<i>MDH1</i>	334	11.29	12.77	2.8	15.5	23
<i>CIT1</i>	479	9.76	13.2	10.8	20.5	28
<i>IDH1</i>	360	11.56	13.14	3.0	16.5	21
<i>KGD1</i>	1014	10.4	12.57	4.5	21	27.5

Graphical abstract



Summary figure

Highlights

- We determine the yeast proteome of fermentation and respiration in diploid cells
- We integrate genome-wide RNA profiling data and large-scale protein detection data
- We identify genes likely regulated at the post-transcriptional level
- We report novel Rpd3/Ume6 target genes
- We show how acetate primes cells to enter sporulation by activation of meiotic genes